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Brain natriuretic peptide is cosecreted with atrial natriuretic peptide from porcine cardiocytes

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Using primary cultures of atrial cardiocytes from neonatal pig, the secretion of brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP)-like immunoreactivities (LI) was studied in vitro. Porcine cardiocytes time-dependently secreted both BNP-LI and ANP-LI into medium under a serum-free condition, although the amount of BNP-LI secreted was about one-third that of ANP-LI. Phorbol ester and Ca^{2+} ionophore had less stimulatory effects on secretion of BNP-LI than that of ANP-LI. Reverse-phase HPLC of the conditioned medium revealed a single major BNP-LI component corresponding to synthetic porcine BNP(1–26). These data suggest that a small molecular weight form BNP, possibly BNP(1–26), is cosecreted with ANP from porcine cardiocytes.

Brain natriuretic peptide; Atrial natriuretic peptide; Cosecretion; Cardiocyte

1. INTRODUCTION

Matsuo and his colleagues have recently isolated a novel natriuretic peptide from the porcine brain [1], designated brain natriuretic peptide (BNP). Porcine (p) BNP consists of 26 amino acid residues with an intramolecular disulfide linkage; pBNP shows a remarkable sequence homology to atrial natriuretic peptide (ANP), and has pharmacological effects similar to those of ANP [1]. Subsequent study has demonstrated that γ-BNP, a large molecular weight form carrying pBNP at its C-terminus, has been identified in porcine cardiac atrium [2]. However, it remains unknown whether BNP is cosecreted with ANP from the porcine heart. Therefore, we have attempted to elucidate the cellular mechanism of BNP secretion from primary cultures of porcine atrial myocytes and to compare this with that of ANP secretion.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Porcine atrial myocytes were prepared by the enzymatic method as described [3]. In brief, both right and left atrium removed from a neonatal pig (1–6-day-old) were dissociated with 0.1% collagenase and pipetted. The same procedure was repeated several times, and the combined cell suspensions were seeded into a gelatinized plastic dish. After incubation at 37°C for 30 min, the non-attached cells predominantly composed of cardiocytes were removed and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum for 48 h. After reaching confluency, the cells were usually incubated in serum-free DMEM at 37°C for 1 h; medium was removed and assayed for immunoreactive (IR)-BNP and IR-ANP.

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2.2. *Radioimmunoassays (RIA) for BNP and ANP*

ANP RIA was performed as previously reported [3], by the use of the rabbit anti-ANP serum (Peninsula Lab., Belmond, CA), which mainly recognizes the common C-terminal region of ANP molecules. ^{125}I-labelled rat (r) ANP (sp. act. 2000 Ci/mmol, Amersham Japan, Tokyo) was used as a tracer, and synthetic α-rANP (Peptide Institute, Osaka) as a standard. BNP RIA was performed as recently reported [4], by the use of the rabbit anti-pBNP serum which does not cross-react with the known ANP molecules. [^{125}I]pBNP was prepared by the lactoperoxidase method and purified by reverse-phase HPLC (sp. act. 500 Ci/mmol). Synthetic pBNP (Peptide Institute) was used as a standard. Separation of the bound ligands from free ligands in both RIAs was accomplished by the double antibody method.

2.3. *Reverse-phase HPLC*

The pooled conditioned media (96 ml) from cultured atrial cardiocytes were acidified with trifluoroacetic acid (TFA) and applied to the Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA), which was eluted with 70% acetonitrile/0.1% TFA. An aliquot of the extracts was subjected to reverse-phase HPLC using an octadecyl-silica column, which was eluted with a linear gradient (15–60%) of acetonitrile in 0.09% TFA for 60 min. Flow rate was 1 ml/min; 1-ml fractions were collected and subjected to RIAs for BNP and ANP.

3. RESULTS

Porcine atrial cardiocytes after 2-day-culture secreted IR-BNP as well as IR-ANP into serum-free medium as a function of time (fig.1): secretion of both

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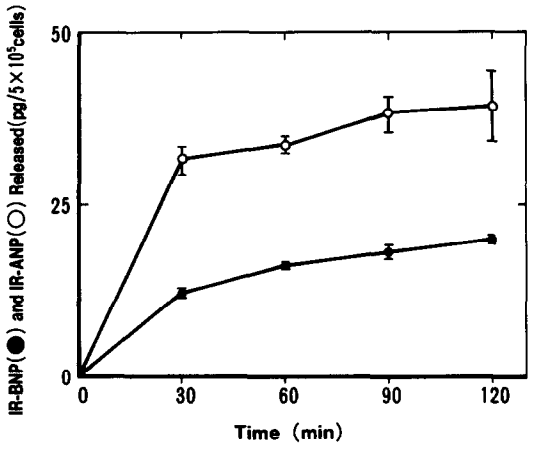

Fig.1. Secretion of BNP and ANP from cultured porcine cardiocytes as a function of time. Time courses of IR-BNP (●) and IR-ANP (○) are shown. Each point is the mean of triplicate dishes; bars show SE.

BNP and ANP became plateau after 60 min, although the amount of IR-BNP released was about one-third that of IR-ANP. The dilution curves of culture medium extracts were parallel to the standard curves of pBNP and rANP in each RIA (fig.2).

The effects of tetradecanoylphorbol acetate (TPA), a protein kinase C (PKC) activator, and Ca^{2+} ionophore

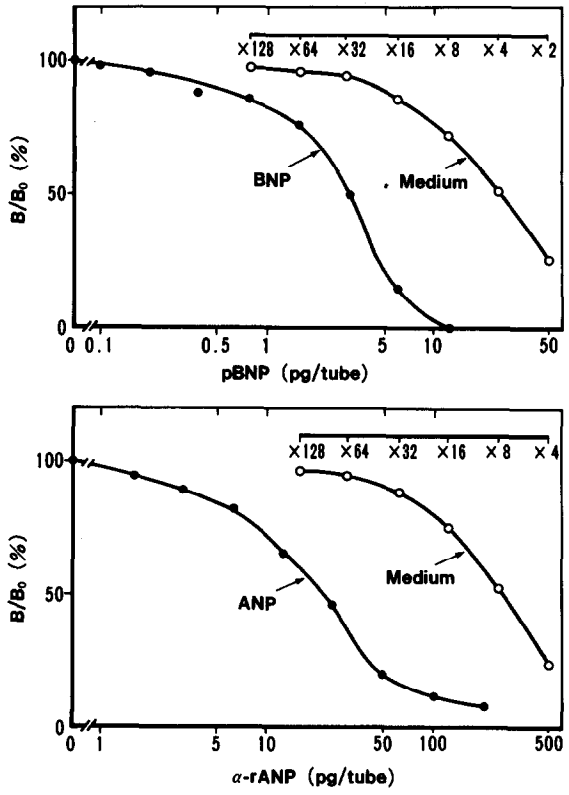

Fig.2. Dilution curves of the media from cultured porcine cardiocytes in BNP and ANP RIAs. Serial dilution curves of the condition media (○) from cultured porcine cardiocytes are compared to those of standard (●) pBNP and α-rANP in BNP RIA (upper panel) and ANP RIA (lower panel), respectively.

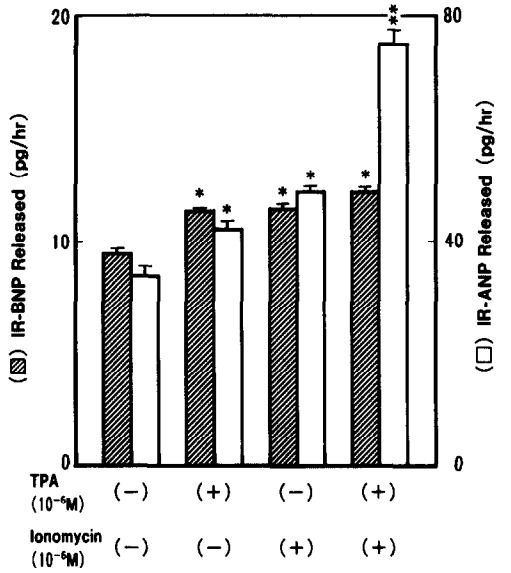

Fig.3. Effects of TPA and ionomycin on secretion of BNP and ANP from cultured porcine cardiocytes. Porcine cardiocytes (5×10^5 cells) were incubated at 37°C for 60 min in the absence and the presence of 10^{-6} M TPA, 10^{-6} M ionomycin, or both. The means of IR-BNP (▨) and IR-ANP (□) released are shown; bars indicate SE (* $P <$ 0.01, ** $P < 0.05$ vs control).

ionomycin on the secretion of IR-BNP and IR-ANP were studied (fig.3). Both TPA and ionomycin significantly stimulated IR-ANP secretion, although they had only slight stimulatory effects on IR-BNP secretion. The combination of both compounds had a synergistic effect on IR-ANP secretion, while their synergistic effect on IR-BNP secretion was minimal.

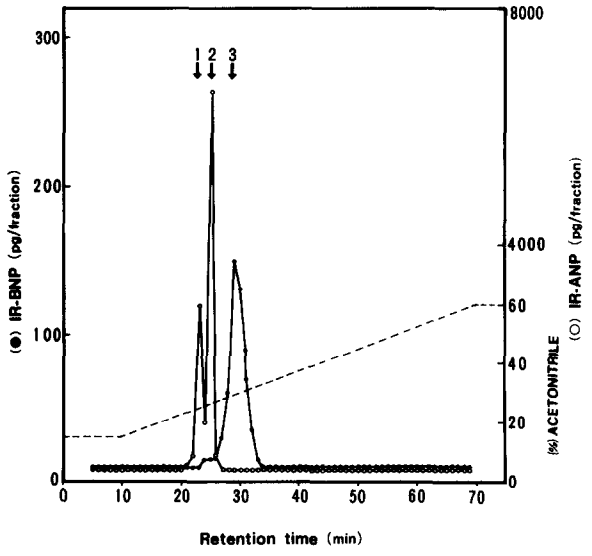

Fig.4. Reverse-phase HPLC profiles of the conditioned media from cultured porcine cardiocytes. Elution profiles of IR-BNP (●) and IR-ANP (○) in the cultured media are shown. Dotted line denotes the linear gradient (15–60%) of acetonitrile. Arrows indicate the elution positions of: (1) α-hANP(5–28); (2) α-hANP(1–28); and (3) pBNP(1–26), respectively.

The reverse-phase HPLC elution profiles of IR-BNP and IR-ANP in extracts of the conditioned media from porcine atrial myocytes are shown in fig.4. A single major IR-BNP component was eluted at a position identical to that of pBNP(1–26). In contrast, two major IR-ANP components eluted earlier than BNP were observed: one component coeluting with hANP(5–28) and the other with hANP(1–28).

4. DISCUSSION

BNP is a novel natriuretic/vasodilatory peptide with a high homology to, but distinct from ANP [1]. Although BNP was originally isolated and sequenced from porcine brain, subsequent studies have demonstrated that γ-BNP, a large molecular weight form carrying BNP at its C-terminus, is also present in the porcine atrium [2], and that a small molecular weight form IR-BNP is secreted into the perfusate from isolated porcine atria [5]. These data suggest that BNP is also synthesized by the heart and secreted into the circulation in a similar fashion to ANP.

The present results clearly show that BNP-like material released from cultured porcine cardiocytes is immunologically similar to standard pBNP, and that both IR-BNP and IR-ANP from cardiocytes time-dependently accumulate in media under a serum-free condition. These data indicate that porcine cardiocytes actually synthesize and secrete IR-BNP concomitantly with IR-ANP. The amount of IR-BNP secreted from cardiocytes is about one-third that of IR-ANP. These data are compatible with those of a recent study showing that IR-BNP levels in the perfusate from isolated porcine heart are much lower than those of ANP [5].

We have recently demonstrated that TPA and ionomycin are potent secretagogues for ANP in cultured rat cardiocytes, and the combination of both compounds has synergistic effect, suggesting that activation of PKC and Ca^{2+} mobilization are closely involved in the secretory mechanism of ANP [3]. The present results of the potent stimulatory effects by TPA and ionomycin as well as their synergistic effect on ANP secretion from porcine cardiocytes are in agreement with our previous observation [3]. In contrast, the stimulatory effects of both compounds on IR-BNP secretion appear to be much less than those of IR-ANP. It should be noted that the cDNA encoding BNP precursor has AT-rich repetitive sequences in the 3′-untranslated region [6], which are known to destabilize mRNA in the cells. Therefore, it is speculated that BNP mRNA transcribed in response to stimuli may be degraded more readily and removed faster from the cardiocytes than ANP mRNA which does not contain such destabilizing sequences [7]. Therefore, it is suggested that BNP and ANP may have different biosynthetic regulatory mechanisms at the transcriptional level.

Reverse-phase HPLC coupled with RIAs for BNP and ANP reveals that IR-BNP in media from porcine cardiocytes consisted of only one major component with identical retention time to that of authentic pBNP(1–26), while IR-ANP consisted of two major components, one coeluting with α-hANP(1–28) and the other with α-hANP(5–28), respectively. Since γ-BNP and γ-ANP are precursors for BNP and α-ANP, respectively, and both function as storage forms in cardiac tissues [2,8], it is suggested that the small molecular weight form BNP, possibly BNP(1–26), is processed from γ-BNP and secreted in analogy to α-ANP from γ-ANP [8]. These data are consistent with those of our recent study showing that the major IR-BNP in human plasma is a small molecular weight form BNP [4]. Taken together, these data strongly suggest that BNP(1–26) is the main secretory form in pigs.

In conclusion, our study demonstrates for the first time that BNP is cosecreted with ANP from porcine cardiocytes, and that a small molecular weight form, possibly BNP(1–26), is the main secretory form.

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